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Pre- and post-translocational stages in protein secretion by *Bacillus subtilis*

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***Proteomic dissection of Signal
Recognition Particle-dependence in
protein secretion by *Bacillus subtilis****

**Geeske Zanen, Haike Antelmann, Rob Meima, Jan D. H. Jongbloed,
Marc Kolkman, Michael Hecker, Jan Maarten van Dijl, and Wim J.
Quax.**

Abstract

In all organisms, the signal recognition particle (SRP) plays an important role in protein transport. The bacterial SRP-dependent pathway is believed to be the major targeting route for membrane proteins, as well as for subsets of secretory proteins. The present studies were aimed at an assessment of the role of two key components of SRP, namely Ffh and FtsY, in protein secretion by *Bacillus subtilis*. Our results show that both components are important for the extracellular accumulation of proteins containing known signal peptides. Remarkably, the extracellular accumulation of individual proteins was affected to different extents by depletion of Ffh or FtsY, at least under the conditions tested. Moreover, the observed Ffh- or FtsY-dependence of certain secretory proteins did not seem to correlate with signal peptide length or hydrophobicity. These findings indicate that other, as yet unidentified, determinants in secretory proteins are also important for their recognition by SRP. High-level production of homologous and heterologous secretory proteins was shown to result in elevated cellular Ffh and FtsY levels. This phenomenon is, most likely, due to post-transcriptional regulation. In conclusion, the present proteomic dissection of SRP-dependent protein secretion provides exciting leads to identify novel determinants for interactions between secretory proteins and SRP.

1. Introduction

In prokaryotes and eukaryotes, the transport of many secretory and membrane proteins to the plasma membrane, chloroplast thylakoids, and the endoplasmic reticulum, is mediated by the signal recognition particle (SRP; Keenan *et al.*, 2001; Lührink and Sinning, 2004; Shan and Walter, 2005). At present, the model of SRP-dependent protein translocation in *B. subtilis* is largely based on observations concerning SRP-dependent translocation in *E. coli*. In this Gram-negative bacterium, a hydrophobic N-terminal signal peptide or transmembrane segment that emerges from the ribosome is believed to be bound by SRP, resulting in the formation of the so-called SRP/RNC (ribosome nascent chain) complex. Next, this complex interacts with the membrane-bound SRP Receptor (FtsY), which assists in the delivery of the SRP/RNC complex to the translocation channel in the membrane. This results in the dissociation of the SRP/FtsY complex. Subsequently, SRP and FtsY can participate in another cycle of protein targeting and translocation (Lührink and Sinning, 2004).

SRP is a protein–RNA complex present in all organisms, but the precise composition varies. The most conserved part of the SRP RNA, domain IV, binds to the 54 kDa component Ffh (Fifty-four homologue) of this complex (Römisch *et al.*, 1989; Bernstein *et al.*, 1989). The Ffh protein is critical for the binding of the targeting signals in secretory and membrane proteins. The hydrophobicity of these targeting signals is crucial for the interaction with Ffh (Valent *et al.*, 1997; de Gier *et al.*, 1998; Chapter 4). Specifically, Ffh consists of three domains; the N domain (α -helical domain), the G domain (nucleotide binding domain), and the M domain (methionine-rich domain, responsible for the interaction with RNA and signal peptide binding) (Lührink and Sinning, 2004). The *B. subtilis* SRP complex consists of the Ffh protein (Honda *et al.*, 1993), a small cytoplasmic RNA (scRNA), and a histone-like protein (HBSu) (Nakamura *et al.*, 1992; 1994; 1999). HBSu is a DNA binding protein (Klein and Marahiel, 2002), which is essential for normal growth of *B. subtilis* (Nakamura *et al.*, 1999). Like Ffh, FtsY belongs to the widely conserved family of SRP-GTPases (Eichler and Moll, 2001). In general, the FtsY protein consists of the N and G domains that are homologous to the N and G domains of Ffh. *E. coli* FtsY also contains a third domain, an acidic domain at the N terminus (A domain), which is homologous to the A domain of the SR α component in the mammalian SRP receptor. Notably, FtsY of *B. subtilis* lacks this A domain (Zanen *et al.*, 2004; Chapter 3).

The processes of protein targeting and transport in *B. subtilis* have attracted wide interest, because this organism has a remarkably large capacity to secrete homologous and heterologous proteins into its external environment (Westers *et al.*, 2004b). A pivotal role in the secretion process has been proposed for the SRP pathway since the signal peptides of *B. subtilis* are generally longer and

more hydrophobic than those of Gram-negative bacteria (Tjalsma *et al.*, 2000; 2004; Chapter 4). Consistent with this view, Hirose *et al.* (2000) have shown by proteomics that a severe depletion of Ffh results in a nearly complete block in the secretion of proteins *via* the Sec machinery of *B. subtilis*. Nevertheless, little is thus far known about the relative importance of Ffh for the secretion of different proteins produced at wild-type or elevated levels. Moreover, the general role of FtsY in the *B. subtilis* secretion process has not been documented. Only a role of FtsY in the assembly of the outer coat proteins of *B. subtilis* spores has, thus far, been reported (Kakeshita *et al.*, 2000). Therefore, the present studies were aimed at gaining more insights in the SRP-dependent protein transport by *B. subtilis*. For this purpose, we investigated the influence of controlled depletion of Ffh or FtsY on the secretion of native proteins produced at wild-type levels by proteomics. In addition, we investigated how the SRP pathway copes with the secretion of overexpressed homologous and heterologous proteins, such as proteases or the lipase LipA of *B. subtilis*, or the α -amylase AmyQ of *Bacillus amyloliquefaciens*. The results of these studies show that the secretion of different signal peptide-containing proteins depends to different extents on Ffh or FtsY. These observations are of general interest, firstly because Ffh and FtsY are essential for growth and viability of *B. subtilis*, and secondly because the process of protein secretion by this Gram-positive bacterium is both of biotechnological and fundamental scientific interest.

2. Materials and Methods

2.1 Plasmids, bacterial strains and media

The plasmids and bacterial strains used are listed in Table 1. TY medium contained Bacto tryptone (1%), Bacto yeast extract (0.5 %), and NaCl (1%). If required, media were supplemented with spectinomycin (Sp; 100 μ g/ml); tetracyclin (Tc; 15 μ l/ml); kanamycin (Km; 10 or 20 μ g/ml); or isopropyl- β -D-thiogalactopyranoside (IPTG; on plate: 10 μ g/ml for *Iffh*; 25 μ g/ml for *IftsY*; in liquid media: 100 μ g/ml for *Iffh*; 250 μ g/ml for *IftsY*). Both *B. subtilis* *Iffh* and *IftsY* were obtained through a Campbell-type chromosomal integration of pMutin2 derived plasmids in upstream sequences of the *ffh* and *ftsY* genes, respectively (Vagner *et al.*, 1998; M. Kolkman, *unpublished results*). For this purpose, these plasmids contained homologous fragments for targeted chromosomal integration and an antibiotic resistance marker for selection in *B. subtilis*. When cells were transferred from TY medium with IPTG to fresh TY medium without IPTG, they were gently washed three times with fresh TY medium without IPTG prior to transfer to fresh TY medium. As a control, an aliquot of the washed cells was also resuspended in fresh TY medium with IPTG.

Table 1. Plasmids and Bacterial Strains

Plasmids	Relevant properties	Reference
pMAP65	Vector for high-level expression of the <i>lacI</i> gene of <i>E. coli</i> ; Km ^r	Petit <i>et al.</i> , 1998
pKTH10	Vector encoding the AmyQ gene of <i>B. amyloliquefaciens</i> ; Km ^r	Palva, 1982
pLip2031	<i>E. coli</i> / <i>B. subtilis</i> vector; encodes the lipase LipA of <i>B. subtilis</i> ; Km ^r	Dartois <i>et al.</i> , 1994
<i>B. subtilis</i> strains	Relevant properties	Reference
168	<i>trpC2</i>	Kunst <i>et al.</i> , 1997
<i>ylxM</i>	<i>trpC2</i> ; in frame mutation in <i>ylxM</i> , Ffh overproduction strain	Laboratory collection Genencor International
DegU(hy)	originally denoted as MD300; <i>trpC2</i> ; <i>degU32</i> (hy), Km ^r	Mäder <i>et al.</i> , 2002
<i>Iffh</i>	<i>trpC2</i> ; <i>ffh</i> ::pMutin2, Sp ^r ; a 798-bp <i>EcoRI</i> / <i>Bam</i> HI fragment of the 5' region of <i>ffh</i> was cloned in pMutin2	Laboratory collection Genencor International
<i>Iffh</i> DegU(hy)	<i>trpC2</i> ; <i>ffh</i> ::pMutin2, <i>degU32</i> (Hy), Km ^r , Sp ^r	This study
<i>IftsY</i>	<i>trpC2</i> ; <i>ftsY</i> ::pMutin2, Tc ^r ; a 517-bp <i>EcoRI</i> / <i>Bam</i> HI fragment of the 5' region of <i>ftsY</i> was cloned in pMutin2	Laboratory collection Genencor International

2.2 DNA and RNA techniques

Procedures for PCR, (chromosomal) DNA purification, ligation and restriction were carried out as described by Sambrook *et al.* (1989). Competent *B. subtilis* cells were transformed as previously described (Tjalsma *et al.*, 1998). The *degU32*(hy) mutation was introduced into the *Iffh* or *IftsY* mutant strains by transformation with chromosomal DNA of *B. subtilis* DegU(hy). During the entire transformation

procedure, the *Iffh* and *IftsY* strains were incubated in the presence of 100 and 250 μ M IPTG, respectively. RNA isolation was performed as described by Campo *et al.* (2004). Using the RevertAid™ First Strand cDNA Synthesis Kit, the production of cDNA was performed according to the instructions of the manufacturer Fermentas (Canada). Real Time PCR was performed as described by Peters *et al.* (2004) using specific primers and custom-made TaqMan probes (Biosearch Technologies, USA).

2.3 SDS-PAGE, Western blotting and immunodetection

To visualise proteins of *B. subtilis* by Western blotting, cells were separated from the growth medium by centrifugation (3 min, 11,800 rpm, room temperature). Cellular samples and growth medium samples of *B. subtilis* were prepared for polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (van Dijl *et al.*, 1991). If necessary, growth medium samples were 20-fold concentrated by trichloroacetic acid (TCA) precipitation and, in parallel, the corresponding cell samples were treated with 5% TCA and concentrated four-fold. After separation by SDS-PAGE, proteins were transferred to a Protran® nitrocellulose transfer membrane (Schleicher and Schuell BioScience, Germany). Western blotting was performed as described by Kyhse-Andersen (1984). AmyQ, Ffh, FtsY, GroEL, and LipA were visualised with specific antibodies and horseradish peroxidase anti-rabbit IgG conjugated or alkaline phosphatase-conjugated anti-rabbit IgG (Biosource International, USA). For visualisation of the horseradish peroxidase conjugate, the ECL⁺ kit (Amersham, United Kingdom) was used and the signal was detected by a ChemiGenius² XE (Syngene, United Kingdom) image acquisition system. The alkaline phosphatase conjugate was detected using a standard Nitro Blue Tetrazolium- 5-bromo-4-chloro-3-indolyl phosphate (NBT-BCIP) reaction (Sambrook *et al.*, 1989).

2.4 AmyQ activity assay

The production of AmyQ by *B. subtilis* was assayed with a plate (halo) assay for α -amylase activity. For this purpose, aliquots of growth medium were spotted on Durapore® membrane filters (Millipore, Ireland) that were placed on TY-agar plates containing 1 % starch (Westers *et al.*, 2003; Chapter 4). *B. subtilis* cells were grown to post-exponential phase. Next, the medium was separated from the cells by centrifugation and spotted on the filters after a correction for OD₆₀₀. After overnight incubation at 37°C, the plates were exposed to iodine vapor and analysed for starch degradation.

2.5 Protease activity assay

To visualise extracellular protease activity, TY agar plates were supplemented with 1% skim milk. Aliquots of overnight cultures were spotted on the agar plates, which

were incubated overnight at 37°C, and finally analysed for skim milk degradation (Bolhuis *et al.*, 2000).

2.6 Two-dimensional gel electrophoresis and image analysis

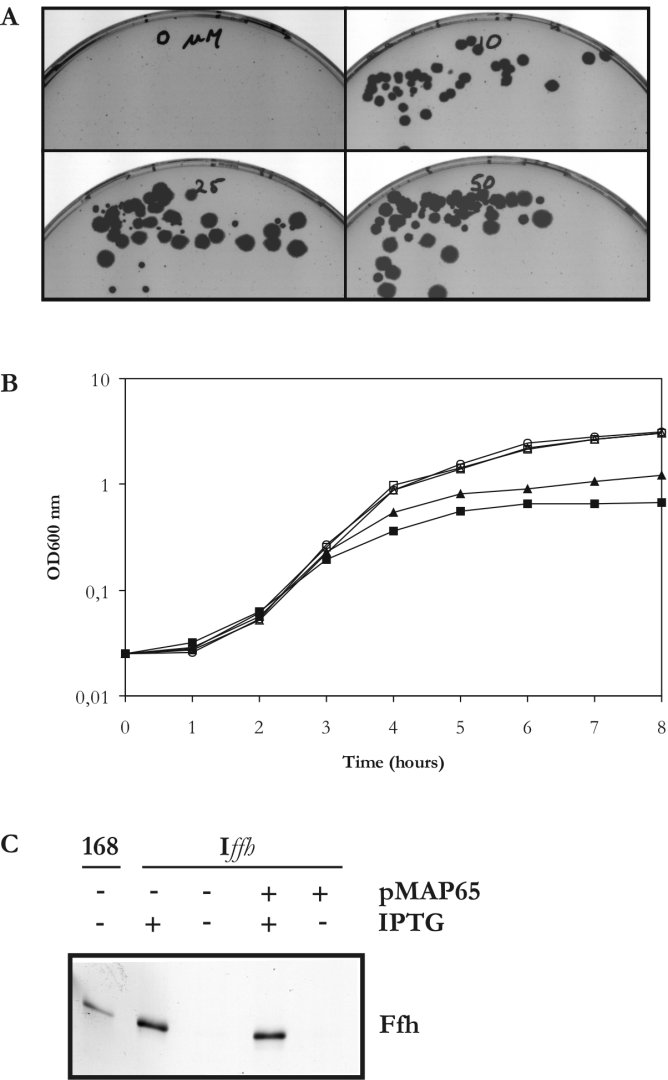
To monitor the effect of Ffh depletion, the composition of the extracellular proteomes of *B. subtilis* Iffh pMAP65 grown in the absence or presence of 100 µM IPTG was compared. To investigate the involvement of FtsY in protein secretion, the composition of the extracellular proteome of the IftsY strain grown in the absence of IPTG was compared with that of the parental strain *B. subtilis* 168. All strains were grown at 37°C under vigorous agitation in 1 liter of TY medium. After 1h of post-exponential growth, cells were separated from the growth medium by centrifugation, and proteins secreted into the growth medium were concentrated by TCA precipitation. The resulting samples were used for two-dimensional gel electrophoresis (2D PAGE). Gels were stained with the SYPRO Ruby protein gel stain (Invitrogen, United Kingdom) or Coomassie brilliant blue, and protein spots were identified by matrix-assisted laser desorption / ionisation – time of flight mass spectrometry (MALDI-TOF MS) and/or N-terminal sequencing as previously described (Antelmann *et al.*, 2001). In some cases, gels were stained with silver nitrate (Blum *et al.*, 1987). To visualise possible differences in extracellular proteome composition, image analysis of gels stained with the SYPRO Ruby protein gel stain was performed using the DECODON Delta 2D software (<http://www.decodon.com>). All quantifications are relative and each spot volume is related to the cumulated spot volumes of the entire gel. Using the DECODON Delta 2D software it is not possible to reach absolute quantification or to use externally applied protein standards for calibration. All ratios are calculated from the % of the specific spot volumes (related to the total spot volumes of the entire gels) of the sample image divided by the master image. Each experiment was performed at least three times.

3. Results

3.1 Proteomics of Ffh depletion

To study the role of Ffh in protein secretion, the *B. subtilis* Iffh strain was used, in which the *ffh* gene is placed under the transcriptional control of the IPTG-inducible Pspac promoter. When grown on TY-agar plates, the Iffh mutant strain was shown to be completely IPTG-dependent (Fig. 1A). Colonies of this mutant had uniform morphologies on agar plates containing 10 µM IPTG, but not at higher IPTG concentrations. This revealed that 10 µM was an optimal concentration of IPTG for unimpaired growth of *B. subtilis* Iffh on plates. In contrast, as shown in Figure 1B, the growth of Iffh cells in liquid TY medium was not strictly IPTG-dependent. Even the introduction of plasmid pMAP65, containing an additional copy of *lacI* for

improved *Pspac* repression, did not result in complete IPTG-dependence. Nevertheless, the growth of the *Iffh* strain containing pMAP65 was severely impaired in the absence of IPTG (Fig. 1B). Notably, the *Iffh* cells that had grown for 8 hours in liquid medium without IPTG were still completely IPTG-dependent on plates (data not shown), indicating the absence of reversions.



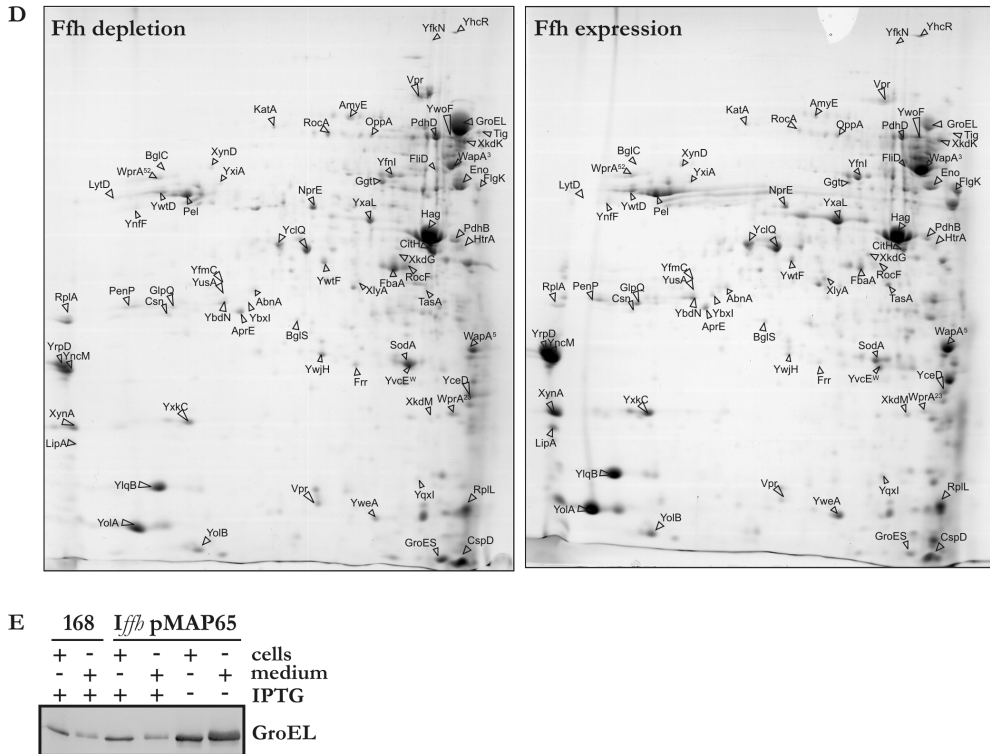


Figure 1. Proteomics of Ffh depletion

A) Phenotype of *B. subtilis* strain *Iffh* grown overnight at 37°C on TY agar plates containing different amounts of IPTG (0, 10, 25, 50 µM). **B)** Growth curves of the *B. subtilis* strains *Iffh* and 168 (parental strain). Cells of overnight cultures grown in the presence of IPTG were collected, gently washed three times with TY medium, diluted till an OD₆₀₀ of 0.025, and subsequently grown and analysed in TY medium with or without IPTG. Samples were withdrawn at hourly intervals. The *B. subtilis* strains used were: 168 (open circles); *Iffh* + IPTG (open triangles); *Iffh* – IPTG (black triangles); *Iffh* pMAP65 (encodes LacI) + IPTG (open squares); *Iffh* pMAP65 – IPTG (black squares). **C)** Cellular levels of Ffh in *B. subtilis* 168 and *Iffh* mutant strains. Samples for Western blotting and immunodetection were withdrawn from cultures grown into stationary growth phase in the presence or absence of IPTG. The cells either contained or lacked plasmid pMAP65. **D)** The extracellular proteome of *B. subtilis* *Iffh* pMAP65. Cells were grown in the presence (Ffh expression) or absence (Ffh depletion) of IPTG until one hour after entry into the stationary phase and extracellular proteins were analysed by 2D gel electrophoresis. Protein spots identified by mass spectrometry and/or N-terminal sequencing are indicated. **E)** Levels of the GroEL protein in cells or medium fractions of *B. subtilis* 168 and *Iffh* pMAP65. Samples for Western blotting and immunodetection were withdrawn from cultures grown into the stationary growth phase in the presence or absence of IPTG. Medium samples were 10x concentrated by TCA precipitation.

To test whether, and under which conditions, the Ffh protein was detectable or absent, samples of the *Iffh* mutant strain and the parental *B. subtilis* strain 168 were analysed by Western blotting and immunodetection with Ffh-

specific antibodies. As shown in Figure 1C, cells of *Iffh* mutant strains with or without pMAP65 produced Ffh when grown in the presence of IPTG. Notably, *Iffh* cells grown in the presence of IPTG contained significantly higher levels of Ffh than the parental 168 strain. Importantly, the amounts of Ffh in *Iffh* cells grown in the absence of IPTG were below the detection level, irrespective of the presence or absence of pMAP65.

The effect of Ffh depletion on protein secretion by *B. subtilis* was investigated by inspection of the extracellular proteome of *Iffh* mutant cells containing pMAP65 grown in the absence of IPTG (Fig. 1D; Ffh depletion), and comparison with the extracellular proteome of these cells grown in the presence of IPTG (Fig. 1D; Ffh expression). Remarkably, most extracellular proteins were still detectable in the growth medium of Ffh-depleted *Iffh* mutant cells and the levels of only a subset of the extracellular proteins were significantly decreased. Table 2 summarises the specific effects of Ffh depletion on the levels of different extracellular proteins (the increases or decreases in the relative spot volumes for each protein are indicated). As expected on the basis of previous results published by Hirose *et al.* (2000), a reduction in the amounts of several signal peptide containing proteins was observed upon Ffh depletion. Specifically, Ffh depletion had a major impact on the extracellular levels of AbnA, Csn, GlpQ, PenP, TasA, and YvcE. These proteins appear to be secreted *via* the Sec pathway of *B. subtilis* (Jongbloed *et al.*, 2002; Tjalsma *et al.*, 2004). Unexpectedly, however, the levels of several other proteins containing signal peptides remained unaffected and the levels of some proteins with signal peptides were even elevated. On balance, the relative extracellular levels of proteins synthesised with signal peptides were predominantly reduced upon Ffh depletion, whereas the relative extracellular levels of proteins without signal peptides were mostly elevated with a few exceptions. These observations imply that Ffh is specifically involved in the secretion of many, but not all, proteins containing a signal peptide.

One of the proteins that were present at strongly elevated levels on the extracellular proteome of Ffh depleted cells was the chaperonin GroEL (Fig. 1D), which is also detectable on the extracellular proteome of the parental strain 168 (Antelmann *et al.*, 2001). This raised the question whether Ffh depletion resulted in an induction of GroEL synthesis and/or release into the growth medium. To address this question, we examined cellular and extracellular levels of GroEL upon Ffh depletion by Western blotting. As shown in Figure 1E, both the cellular and extracellular GroEL levels were significantly increased due to Ffh depletion upon growth of *B. subtilis Iffh* pMAP65 in the absence of IPTG. In contrast, the cellular and extracellular levels of GroEL produced by *B. subtilis Iffh* pMAP65 grown in the presence of IPTG were comparable to those produced by the parental strain 168. This conclusion was confirmed by Western blotting of total samples from cultures of *B. subtilis Iffh* pMAP65 or 168 grown in the presence or absence of IPTG (data

Table 2. Extracellular Proteome Composition^{a)}

Impact Ffh depletion ^{b)}	Proteins with export signal ^{c)}	Proteins without known export signal
Reduced protein levels	AbnA (9,8), Csn (20,0), GlpQ (33,3), LipA (2,8), Pel (2,0), PenP (13,2), TasA (50,0), WapA-3 (5,5), WapA-4 (3,7), XlyA (2,2), XynA (2,5), XynD (4,5), YbdN (1,8), YbxI (6,3), YfkN (1,8), YncM (3,0), YnfF (1,6), YvcE (333,3), YweA (3,8), YxaL (2,7), YxkC (1,7)	XkdG (2,0)
No significant change	AprE, BglS, Vpr, YclQ-1, YclQ-2, YlqB, YoaA, YwoF, YwtD, YxiA	XkdK, YoaB
Elevated Protein levels	Ggt (3,0), HtrA (2,0), NprE (1,7)	CitH (3,6), Eno (1,6), FbaA (3,1), Frr (2,4), GroEL (6,1), GroES (3,1), Hag (1,5), KatA (1,6), PdhB (2,1), PdhD (1,8), RplA (5,4), RplL (1,5), SodA (3,1), Tig (1,7), YceD (3,4), YwjH (3,8)
Non-conclusive	AmyE, CspD, LytD, OppA, WapA-5, WprA23, YfmC, YhcR, YqxI, Yusa, YwtF	FlgK, FliD, RocA, RocF, XkdM

^{a)} All listed proteins were identified by 2D-PAGE and subsequent MALDI-TOF mass spectrometry and/or N-terminal amino acid sequencing as summarised by Tjalsma et al. (2004).

^{b)} Summary of results shown in Figure 1D. The impact of Ffh depletion is categorised as follows: proteins of which the levels in the extracellular proteome of the *B. subtilis* Iffh pMAP65 strain are reduced when grown in the absence of IPTG (reduced protein levels), proteins for which no significant change in their extracellular levels can be observed (no significant change), proteins of which the levels in the extracellular proteome of Ffh-depleted cells are elevated (elevated protein levels), and proteins that could not be unambiguously evaluated (non-conclusive). Changes in the relative spot volumes of identified extracellular proteins were measured, and 1.4-fold increases or decreases were considered significant. The respective fold-factors for increases or decreases of particular extracellular proteins are indicated between brackets.

^{c)} Identified export signals are Sec-type signal peptides, potential twin-arginine signal peptides, lipoprotein signal peptides, and transmembrane domains.

not shown). These observations imply that Ffh depletion triggers a stress response that results in the synthesis of GroEL at elevated levels and an apparently proportional increase in the release of GroEL into the growth medium.

3.2 Impact of secretory protein production on Ffh

To investigate whether the production of secretory proteins at elevated levels impacts on the production of Ffh, the *degU32(hy)* mutation was introduced in the *Iffh* mutant strain. This *degU* mutation is known to strongly enhance the synthesis and secretion of extracellular proteases (Antelmann *et al.*, 2001; Dahl *et al.*, 1992). As shown in Figure 2A, the activity of these extracellular proteases can be visualised after growth on skim milk-containing TY-agar plates. Higher levels of extracellular protease activity result in increased degradation of casein and, consequently, larger halos on the skim milk plates. Remarkably, the presence of the *degU32(hy)* mutation resulted in elevated Ffh levels, both in the parental strain 168 and the *Iffh* mutant (Fig. 2B). A similar increase of cellular Ffh levels was observed upon overexpression of a plasmid-borne copy of *lipA* of *B. subtilis* in the parental strain 168 and the *Iffh* mutant, which results in a significant overproduction of the lipase LipA by both strains (data not shown).

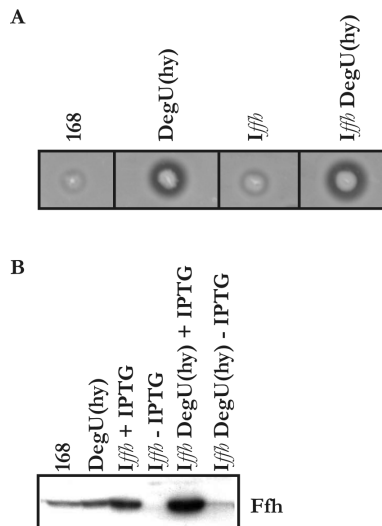


Figure 2. Impact of elevated levels of secretory protein production on cellular Ffh levels

A) Activity of extracellular proteases, produced by *B. subtilis* strains 168, DegU(hy), *Iffh* (grown in the presence of IPTG), or *Iffh* DegU(hy) (grown in the presence of IPTG) was visualised after growth on skim milk-containing TY-agar plates. Colonies were grown overnight at 37°C. **B)** Cellular levels of Ffh in cells of *B. subtilis* strains 168, DegU(hy), *Iffh* (grown with or without IPTG), and *Iffh* DegU(hy) (grown with or without IPTG). Cells were grown until stationary growth phase. Next, samples were collected and used for SDS-PAGE, Western blotting, and immunodetection with Ffh-specific antibodies.

To study the impact of secretory protein overproduction in more detail, the heterologous α -amylase AmyQ of *B. amyloliquefaciens* was used. As depicted in Figure 3A, the cellular level of Ffh increased when cells expressed the plasmid pKTH10-encoded *amyQ* gene, especially in the *Iffh* mutant background. Interestingly, the cellular Ffh level in the *Iffh* cells appeared to be inversely correlated with the presence of AmyQ precursors (Fig. 3B): when Ffh was produced no AmyQ precursors could be observed in the cells, but when the cells were Ffh-depleted AmyQ precursors started to accumulate. Nevertheless, elevated cellular levels of Ffh did not result in the secretion of AmyQ at elevated levels, as evidenced with an AmyQ activity assay (Fig. 3C). On the other hand, however, the cellular depletion of Ffh due to the growth of the *Iffh* mutant strain in the absence of IPTG resulted in the secretion of a reduced amount of active AmyQ compared to the parental strain or *Iffh* mutant cells grown in the presence of IPTG (Fig. 3C). It has to be noted here that cellular Ffh levels were always increased upon the overexpression of the secretory proteins tested (*i.e.* extracellular proteases, LipA, or AmyQ), but the extent to which this happened was found to vary in independent experiments. Also, a certain degree of variation in the cellular pre-AmyQ levels was observed under conditions of induced or repressed Ffh synthesis in *B. subtilis* *Iffh*. Therefore, we tested the impact of constitutive Ffh overproduction on AmyQ production in a *ylxM* mutant strain. The *ylxM* gene precedes the *ffh* gene, and specifies a potential DNA or RNA binding protein of unknown function. An in frame mutation in *ylxM* resulted in a ~five-fold increased Ffh level (Fig.3D), even though the levels of *ffh* mRNA in this mutant were comparable to those in the parental strain 168 (shown by RT-PCR). Consistent with the results obtained for the *Iffh* mutant cells grown in the presence of IPTG, the elevated levels of Ffh in the *ylxM* mutant did not result in an increased extracellular accumulation of active AmyQ (Fig. 3E).

Taken together, these findings show that high-level production of secretory proteins results in elevated cellular levels of Ffh. However, elevated cellular levels of Ffh do not result in increased extracellular levels of secretory proteins.

3.3 Proteomics of FtsY depletion

Like Ffh, FtsY is also essential for cell growth and viability in *B. subtilis* (Oguro *et al.*, 1995) and interacts functionally and physically with the SecYEG translocon in *E. coli* (Angelini *et al.*, 2005). To study the role of FtsY of *B. subtilis* in protein secretion, the *ftsY* gene was placed under the transcriptional control of the IPTG-inducible *Pspac* promoter using plasmid pMutin2. When grown on TY-agar plates, the *IftsY* mutant strain was shown to be completely IPTG-dependent and colonies of this *IftsY* mutant showed a uniform morphology on agar plates containing 25 μ M IPTG (data not shown). This revealed that 25 μ M was an optimal concentration of IPTG for unimpaired growth of *B. subtilis* *IftsY* on plates. In contrast, the growth of

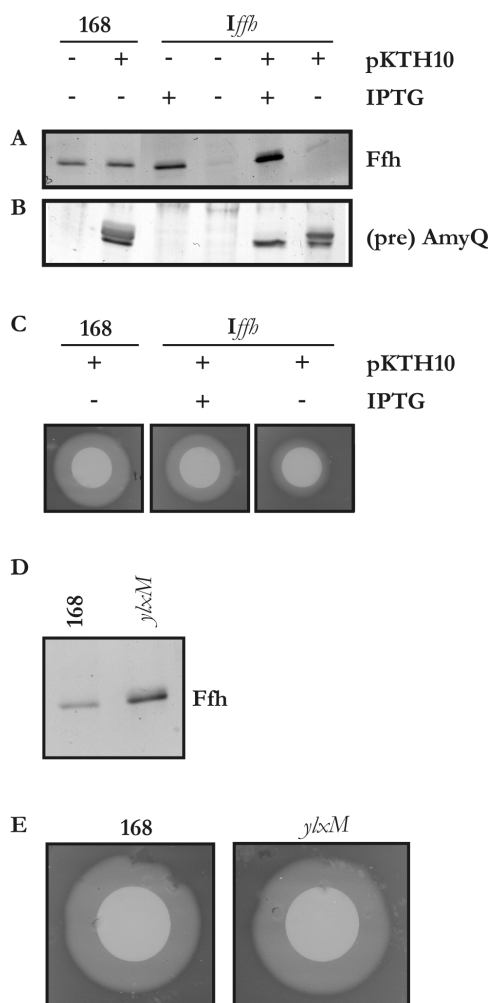


Figure 3. Impact of high-level AmyQ production on cellular Ffh levels

Cellular levels of Ffh (**A**) or AmyQ (**B**) in cells of the *B. subtilis* strains 168 or *Iffh* grown in the absence or presence of IPTG. If required, the cells contained plasmid pKTH10 for AmyQ production. Cells were grown until stationary growth phase, and samples were collected and used for SDS-PAGE, Western blotting, and immunodetection with Ffh- or AmyQ-specific antibodies. **C**) Plate assay for secretion of active AmyQ. Growth medium fractions of *B. subtilis* strains 168 and *Iffh*, which were transformed with plasmid pKTH10, were spotted on TY plates containing 1% starch. Plates were analysed for starch degradation after overnight incubation at 37°C in the presence or absence of IPTG. **D**) Cellular Ffh levels in *B. subtilis* strains 168 and *ylxM*. Cells were grown until stationary growth phase, and samples were collected and used for SDS-PAGE, Western blotting, and immunodetection with Ffh-specific antibodies. **E**) Plate assay for the secretion of active AmyQ. Growth medium fractions of *B. subtilis* strains 168 and *ylxM*, which were both transformed with plasmid pKTH10, were spotted on TY plates containing starch, and incubated overnight at 37°C.

IftsY cells in liquid TY medium turned out to be IPTG-independent even if the cells contained plasmid pMAP65 for LacI overproduction (Fig. 4A). To check whether this growth in liquid medium was due to a loss of IPTG-dependent *Pspac* activity, samples of cells grown for 8 hours were transferred to fresh TY plates with or without IPTG. In marked contrast to the *Iffh* mutant strain, *IftsY* mutant cells had lost their IPTG-dependence during growth in liquid medium. Consistently, low levels of FtsY were detectable by Western blotting analyses of *IftsY* cells grown in liquid medium without IPTG (Fig. 4B). Notably, the FtsY levels in these cells were significantly reduced compared to those observed in cells that were grown in the presence of IPTG, or the parental strain 168. It was therefore concluded that the *IftsY* mutant cells grown in liquid medium could be used to analyse the effects of FtsY depletion on the composition of the extracellular proteome of *B. subtilis*. As shown in Figure 4C, this degree of FtsY depletion resulted in significantly reduced extracellular levels of many secretory proteins containing signal peptides. Table 3 lists the effects of FtsY depletion on the relative extracellular amounts of these proteins. Interestingly, the extracellular levels of different secretory proteins were reduced to different extents upon FtsY depletion. Similar to what was shown for Ffh depletion, the depletion of FtsY resulted in increased extracellular levels of various proteins lacking a known export signal, including GroEL. Taken together, these observations show for the first time that FtsY is involved in the secretion of many, but not all, proteins by *B. subtilis*.

3.4 Impact of secretory protein production on FtsY

To investigate the impact of secretory protein production on cellular FtsY levels, Western blotting experiments were performed. Whereas the Ffh level was increased in a DegU(hy) mutant strain, the cellular level of FtsY was not affected by the *degU32(hy)* mutation (data not shown). Nevertheless, the levels of FtsY were significantly increased by overexpression of plasmid-borne copies of *lipA* (data not shown) or *amyQ* (Fig. 4D) in *B. subtilis* *IftsY* mutant cells. This effect was not observed in cells of the parental strain 168 that were in the post-exponential growth stage after 8 hours of cultivation (Fig. 4E, T=8). However, in exponentially growing cells of *B. subtilis* 168, the expression of AmyQ did result in increased levels of FtsY (Fig. 4E, T=4). Notably, the effect of AmyQ production on the FtsY level in *B. subtilis* *IftsY* was so strong that cells of this mutant, grown in the absence of IPTG, contained about wild-type amounts of this protein (Fig. 4D). Neither in the *IftsY* strain, nor the parental strain, did the increased levels of FtsY affect the ratio between precursor and mature AmyQ in the cells (Fig. 4, D and E). Finally, whereas the Ffh levels were increased in the *ylxM* mutant strain (Fig. 3D), the FtsY levels in this strain were not detectably different from those in the parental strain (data not shown). These observations show that a five-fold increased Ffh level does not affect the FtsY level.

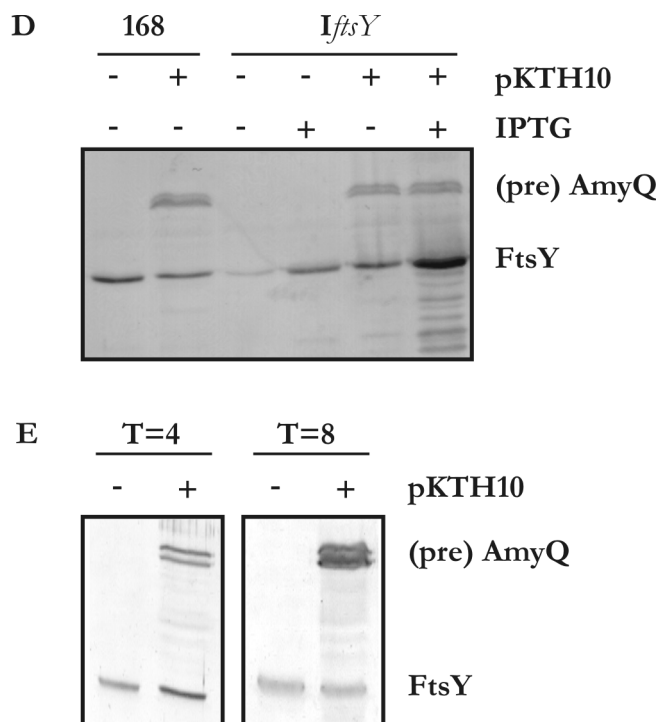


Figure 4. Proteomics of FtsY depletion

A) Growth curves of *B. subtilis* strain *IftsY* pMAP65 grown in TY medium in the absence (black squares) or in the presence of IPTG (open squares). Cells of overnight cultures grown in the presence of IPTG were collected and diluted till an OD600 of 0.025 in fresh TY medium with or without IPTG after gently washing the cells with fresh medium without IPTG. Subsequently, growth was continued for the period of time indicated. Samples were withdrawn at hourly intervals. **B)** Cellular FtsY levels in *B. subtilis* strains 168 and *IftsY* grown in the presence or absence of IPTG. Cells were grown until stationary growth phase. Subsequently, samples were collected and used for SDS-PAGE, Western blotting, and immunodetection with FtsY-specific antibodies. **C)** The extracellular proteomes of *B. subtilis* strain *IftsY* grown in the absence of IPTG (FtsY depletion) and the parental strain 168 (FtsY expression) as analysed by 2D-gel electrophoresis. Cells were grown until one hour after entry into the stationary phase. Protein spots identified by mass-spectrometry and/or N-terminal sequencing are indicated. **D)** Cellular levels of FtsY and (pre-)AmyQ in cells of *B. subtilis* strains 168 and *IftsY* grown in the absence or presence of IPTG. If appropriate, cells were transformed with plasmid pKTH10. Cells were grown until stationary growth phase. Samples were collected and used for SDS-PAGE, Western blotting, and immunodetection with FtsY- and AmyQ-specific antibodies. **E)** Cellular levels of FtsY and (pre-)AmyQ in *B. subtilis* 168 transformed with pKTH10 for AmyQ production. Cells were grown until exponential growth phase (T=4) or stationary growth phase (T=8). Samples were collected and used for SDS-PAGE, Western blotting, and immunodetection with FtsY- and AmyQ-specific antibodies.

Table 3. Extracellular Proteome Composition^{a)}

Impact FtsY depletion ^{b)}	Proteins with export signal ^{c)}	Proteins without known export signal
Reduced protein levels	AbnA (2,0), AmyE (1,5), BglS (1,6), Csn (1,7), GlpQ (2,4), LipA (28,6), LytD (1,6), Mpr (2,5), Pel (2,8), TasA (23,8), Vpr (2,5), WapA-3 (2,5), WprA23 (2,2), XynA (1,9), XynD (2,1), YbdN (1,7), YbfO (2,1), YbxI (3,7), YclQ-1 (3,1), YclQ-2 (2,9), YlqB (2,2), YncM (1,6), YnfF (1,9), YvcE-2 (1,7), YwaD (4,4), YweA (1,5), YwtD (3,4), YxiA (3,0)	YolB (1,6)
No significant change	NprE, OppA, PenP, WprA52, YfmC, YfnI, YolA, YwoF, YxaL	Hag, XkdG, XkdM
Elevated Protein levels	Ggt (2,8), HtrA (2,1)	CitH (2,8), Eno (4,2), FbaA (3,3), Frr (3,8), GroEL (7,9), GroES (11,3), PdhB (2,9), PdhD (3,8), RocA (2,9), RplA (4,8), RplL (8,2), SodA (3,6), Tig (6,9), YwjH (3,1)
Non-conclusive	AprE, BglC, Bpr, Epr, FeuA, MntA, MsmE, PbpX, WapA-4, WapA-5, XlyA, YfkN, YhcR, YoaW, YodJ, YurI, YvcE-1, YwtF, YxeB, YxkC	FlgK, FliD, KatA, MutS, RocF, YceD, XkdM, XepA

^{a)} All listed proteins were identified by 2D-PAGE and subsequent MALDI-TOF mass spectrometry and/or N-terminal amino acid sequencing as summarised by Tjalsma et al. (2004).

^{b)} Summary of results shown in Figure 4C. The impact of FtsY depletion is categorised as follows: proteins of which the levels in the extracellular proteome of the *B. subtilis* IftsY pMAP65 strain are reduced when grown in the absence of IPTG (reduced protein levels), proteins for which no significant change in their extracellular levels can be observed (no

significant change), proteins of which the levels in the extracellular proteome of FtsY-depleted cells are elevated (elevated protein levels), and proteins that could not be unambiguously evaluated (non-conclusive). Changes in the relative spot volumes of identified extracellular proteins were measured, and 1.4-fold increases or decreases were considered significant. The respective fold-factors for increases or decreases of particular extracellular proteins are indicated between brackets.


◊ Identified export signals are Sec-type signal peptides, twin-arginine signal peptides, lipoprotein signal peptides, and transmembrane domains.

4. Discussion

B. subtilis and related bacilli are able to secrete proteins in high amounts into the growth medium and are therefore commercially used for high-level protein production. Here we show that the SRP pathway plays an important role in protein secretion by *B. subtilis*. While previous studies indicated that a severe depletion of Ffh resulted in a nearly complete block of Sec-dependent protein secretion (Hirose *et al.*, 2000), the present studies reveal that Ffh limitation can affect the secretion of Sec-dependent proteins to different extents. A second novel observation was that limitation of the potential SRP-receptor FtsY resulted in a similar defect in protein secretion. Unexpectedly, the high-level production of homologous and heterologous secretory proteins was shown to result in elevated levels of Ffh and, in some cases, FtsY. Nevertheless, elevated levels of Ffh do not seem to result in elevated levels of protein secretion under the conditions tested.

Pioneering studies of Hirose *et al.* (2000) revealed a major block in protein secretion upon Ffh depletion. Even though we depleted Ffh to levels below the detection limit, this did not result in a comparable secretion block. At present, we can only speculate about the possible reasons for this difference. Clearly, Hirose *et al.* (2000) used minimal media for their studies, whereas a rich medium was used in the present studies. The rationale for using a rich medium was that significantly more extracellular proteins are detectable when cells are grown in rich media than when they are grown in minimal media (Antelmann *et al.*, 2001; Tjalsma *et al.*, 2004). Possibly, Ffh depletion is more effective when cells are grown in minimal media. If so, this would relate to the Ffh-dependent secretory and membrane proteins synthesised under these growth conditions. Alternatively, the strain used by Hirose *et al.* (2000) may allow a more drastic depletion of Ffh than the presently used *Iffh* strain. Unfortunately, this possibility will be difficult to verify, because in our *Iffh* strain grown in the absence of IPTG, Ffh is already depleted to levels below the current detection limit.

Previous studies in *E. coli* have shown that signal peptides directing proteins into the SRP-dependent pathway for protein export are significantly more hydrophobic than those mediating SRP-independent targeting (Lee and Bernstein, 2001; Valent *et al.*, 1997). This seems to be true also in *B. subtilis*, as was shown for




AmyQ constructs containing engineered signal peptides with different hydrophobicities (Chapter 4). Consistent with this view, the extracellular levels of GlpQ and WapA, which have highly hydrophobic signal peptides, were reduced significantly upon Ffh depletion. Specifically, the signal peptides of GlpQ and WapA have hydrophobic core domains with average hydrophobicities of 2.3, which is well above the average hydrophobicity of 1.8 observed for such domains in *B. subtilis* signal peptides (van Dijl *et al.*, 2001). In general, however, there seems to be no clear correlation between the extent to which the extracellular level of particular proteins is reduced upon Ffh depletion and the hydrophobicity of the signal peptides of the respective proteins. For example, the secretion of BglS and YwtD, which have highly hydrophobic signal peptides (core domain hydrophobicity of 2.3), appears not to be affected by the depletion of Ffh. Conversely, the secretion of AbnA, PenP, and YvcE appears to be strongly affected by Ffh depletion despite the fact that the signal peptides of these proteins have hydrophobic core domains with average hydrophobicities of 1.4 or even lower. This suggests that signal peptide hydrophobicity is not the only factor that determines Ffh-dependence of a secretory protein. Likewise, no clear correlation between the Ffh dependence of extracellular proteins and the length of their signal peptides could be identified (our unpublished observations). In this respect, it is important to bear in mind that the present proteomic analyses reveal differences in the steady-state levels of extracellular proteins in relation to Ffh depletion, but not the effects on the kinetics of the secretion of these proteins. Clearly, Ffh depletion may have different effects on the rates of secretion of the various extracellular proteins identified in this study. In fact, some of the observed effects may be indirectly caused by Ffh depletion. For example, it is conceivable that Ffh depletion impacts on the functionality of the Sec machinery or certain catalysts for post-translocational folding of exported proteins (Sarvas *et al.*, 2004; Tjalsma *et al.*, 2000). In view of the differential effects of Ffh depletion on Sec-dependent extracellular proteins of *B. subtilis*, we consider an indirect effect through malfunction of the Sec machinery less likely, but this possibility can not be excluded on the basis of our proteomics experiments. Similar considerations are relevant in the evaluation of the observed effects of FtsY depletion. Also in this case, no clear correlation between reduced extracellular accumulation of different proteins and the hydrophobicity or length of their signal peptides was detectable, as underscored by the observed reduction of LipA (average signal peptide hydrophobicity of 1.2) in the medium of FtsY-depleted cells.

Both upon depletion of Ffh or FtsY, the extracellular levels of various proteins are increased. These proteins include mainly proteins that reach the growth medium without a known export signal, many of which have a presumed function in the cytoplasm (*e.g.* GroEL, GroES, and Tig). Such proteins may be released into the growth medium by cell lysis, but an active transport process can

not be excluded at present. Notably, the total cellular levels of the chaperonin GroEL are significantly increased upon Ffh or FtsY depletion, suggesting that a cytoplasmic accumulation of secretory precursor or membrane proteins under these conditions results in a titration of GroEL and, consequently, an induction of the *groEL/groES* operon (Reischl *et al.*, 2002). Interestingly, GroEL has been implicated in protein targeting in *E. coli* (Bochkareva *et al.*, 1988; Kusukawa *et al.*, 1989), but such a function could never be shown for this protein in *B. subtilis* (Tjalsma *et al.*, 2000). Nevertheless, it is tempting to speculate that GroEL is capable of partially replacing the *B. subtilis* SRP. This could be the reason why depletion of Ffh or FtsY in liquid media did not result in a complete growth inhibition. Additionally, the elevated levels of the cytoplasmic chaperone trigger factor (Tig) might be involved in this possible suppression of growth defects, as it is known to compete with SRP for polypeptides that emerge from the *B. subtilis* ribosome (Chapter 4). This would be in line with the idea that the trigger factor could be involved in the export of secretory proteins in certain Gram-positive bacteria, such as *Streptococcus pyogenes* (Lyon *et al.*, 1998).

In addition to the proteins lacking a known export signal, a few proteins synthesised with a signal peptide were detected at increased levels in the growth medium of Ffh- or FtsY-depleted cells (Ggt and HtrA). The mechanism behind this increase is presently not understood, especially in the case of the gamma-glutamyltranspeptidase Ggt. We can speculate, however, in the case of HtrA. Increased extracellular levels of the HtrA protein have, so far, been demonstrated under two different conditions. Firstly, a so-called protein secretion stress response results in increased levels of *htrA* transcription and a concomitant increased release of HtrA into the growth medium (Antelmann *et al.*, 2003; Hyyrylainen *et al.*, 2005). This stress response is correlated with the accumulation of misfolded proteins at the membrane-cell wall interface (Sarvas *et al.*, 2004). Secondly, the extracellular HtrA level is significantly increased upon deletion of multiple extracellular proteases (Antelmann *et al.*, 2003). The latter is due to reduced extracellular degradation of HtrA. Our present observations indicate that, at least upon FtsY depletion, the levels of certain extracellular proteases are reduced (Mpr, Vpr, and WprA), which might result in increased levels of extracellular HtrA. Whether Ffh or FtsY depletion elicits a secretion stress response that leads to increased *htrA* expression remains to be investigated.

High level production of homologous or heterologous secretory proteins was shown to result in increased cellular levels of Ffh. Although the mechanism underlying this effect is presently not entirely clear, various lines of evidence indicate that the Ffh level is increased due to a post-transcriptional event. Firstly, the *ffh* mRNA levels of the parental strain 168 and the DegU(hy) strain were shown to be similar (U. Mäder and G. Homuth, personal communication), whereas the DegU(hy) strain contains a clearly increased amount of Ffh protein. Secondly, high-



level AmyQ production did not result in increased *ffh* mRNA levels (Hyrylainen *et al.*, 2005). Thirdly, high-level production of AmyQ or LipA in the *Iffh* strain, did not result in increased expression of the transcriptional *ffh-lacZ* gene fusion that is present in this strain (data not shown). Finally, the increased Ffh levels upon high-level production of secretory proteins was both observed in strains in which *ffh* transcription was controlled by the native promoter, and in strains in which *ffh* transcription was controlled by the *Pspac* promoter. Possibly, the elevated levels of precursor proteins that interact with Ffh can result in reduced levels of proteolytic turn-over of Ffh, resulting in higher levels of this protein in the cell. However, alternative regulatory mechanisms at the level of Ffh translation can presently not be excluded. Possibly, the increased level of Ffh can help the cell to survive the potentially detrimental effects of high-level protein secretion on, for example, membrane protein biogenesis, which will also depend on Ffh. Whether the increased Ffh level upon production of secretory proteins is required for the efficient secretion of these proteins is not known. In any case, the present studies show that increased cellular levels of Ffh, due to IPTG-induced overexpression of *ffh* or a mutation in *ylxM*, do not result in increased extracellular levels of secreted AmyQ. This implies that the amounts of Ffh in the parental strain 168 producing AmyQ do not set a limit to the secretion of this protein. Compared to Ffh, the conditions that lead to increased levels of FtsY are more restricted. In fact, this was specifically observed in exponentially growing cells of *B. subtilis* 168 producing AmyQ and in the *IftsY* strain producing AmyQ. Also in this case, the effects of secretory protein production occur most likely at the post-transcriptional level, because AmyQ production did neither affect *ftsY* mRNA levels in *B. subtilis* 168 (Hyrylainen *et al.*, 2005) nor the expression of the transcriptional *ftsY-lacZ* gene fusion in *B. subtilis* *IftsY* (data not shown). As indicated above for Ffh, the precise mechanism underlying the increased FtsY levels of cells secreting proteins at high levels remains to be elucidated. The present results do, however, rule out the possibility that Ffh is involved in this mechanism.

Taken together, our observations indicate that both Ffh and FtsY play important roles in the export of Sec-dependent extracellular proteins of *B. subtilis*. A major question that remains to be answered is what determines Ffh- or FtsY-dependence of a secretory protein. Clearly, signal peptide length and hydrophobicity are not the only determinants that are involved in SRP-dependent secretion. The SRP-dependent proteins identified in the present studies may represent suitable leads for further investigations on the role of, for example, signal peptide-mature protein combinations in interactions of secretory precursor proteins with SRP components.

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